Identification of a Novel Compound with MITF Suppression Activity and Apoptosis Induction Activity in Combination with BRAF Inhibitors

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Abstract

Treatment of melanoma with BRAF inhibitors and immune checkpoint inhibitors is associated with a high response rate. However, a subset of melanoma patients with intrinsic and acquired resistance is insensitive to these therapeutics. Thus, to improve melanoma therapy, new drug candidates with novel mechanisms of action need to be identified. The objective of this study was to identify an anti-melanoma agent and to identify the mechanisms by which it inhibits tumor cell growth.

Chapter I describes the identification of an anti-melanoma compound, CH5552074, and its mechanisms of action. In order to identify novel anti-melanoma compounds, a chemical library was screened against a melanoma cell line panel. CH5552074 showed remarkable cell growth inhibition activity in melanoma cell lines. To investigate the mechanisms of action of CH5552074, I conducted an Ingenuity Pathway Analysis (IPA) correlating data on compound sensitivity and gene expression profiles of each cell line. The IPA results suggested that CH5552074-sensitive cell lines had activated microphthalmia-associated transcription factor (MITF), which plays a critical role in melanoma progression. Further *in vitro* studies using the melanoma cell line SK-MEL-5 revealed that suppression of MITF with siRNA resulted in cell growth inhibition. These results showed that CH5552074 inhibited cell growth by reducing the protein expression of MITF. These data support the therapeutic potential of CH5552074 as an anti-melanoma agent with capacity to downregulate MITF protein expression levels.

Chapter II provides information on an orally available compound, CH6868398, which was identified and evaluated for *in vivo* efficacy alone and in combination with a BRAF inhibitor, PLX4720. CH6868398 has a novel chemical structure and arrests MITF protein expression in melanoma cells. It inhibited the growth of MITF-dependent melanoma cells both with and

without BRAF mutation and exhibited anti-tumor effects in a melanoma xenograft model. Because selective BRAF inhibitors are standard therapeutics for BRAF-mutated melanoma, I investigated the effect of CH6868398 in combination with PLX4720 on cell growth inhibition. The addition of CH6868398 enhanced the growth inhibition activity of PLX4720 in melanoma cell lines. Furthermore, the combination of CH6868398 and PLX4720 efficiently suppressed MITF protein expression and enhanced the cleavage of Caspase 3 and poly (ADP-ribose) polymerase (PARP) in melanoma cell lines.

In conclusion, I identified some anti-melanoma agents with a novel mechanism of action, particularly MITF suppression. CH6868398 inhibited tumor growth in a xenograft model and improved the sensitivity of melanoma cells to a BRAF inhibitor. These data suggest the therapeutic potential of CH6868398 as an anti-melanoma agent that reduces MITF protein expression in combination with BRAF inhibitors.

Abbreviations

ATN1:	atrophin-1
CTLA-4:	cytotoxic T-lymphocyte associated protein 4
DMSO:	dimethyl sulfoxide
FDA:	Food and Drug Administration
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
GC-RMA:	guanine-cytosine robust multiarray analysis
HDAC:	histone deacetylase
HIV1:	human immunodeficiency virus type 1
IPA:	Ingenuity Pathway Analysis
MAPK:	mitogen-activated protein kinase
MHC:	major histocompatibility complex
MEK:	MAPK/ERK kinase
MITF:	microphthalmia-associated transcription factor
PARP:	poly (ADP-ribose) polymerase
PAX3:	paired box gene 3
PCR:	polymerase chain reaction
PD-1:	programmed cell death protein 1
RT-PCR:	real-time PCR
PI3K:	phosphoinositide 3-kinase
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLC45A2:	solute carrier family 45 member 2
SOX10:	SRY-related HMG-box 10
SUMO:	small ubiquitin-like modifier
TMB:	tumor mutation burden
TRIM24:	tripartite motif-containing 24
TRPM1:	transient receptor potential cation channel, subfamily M, member 1
UV:	ultraviolet

General Introduction

Melanoma is a highly aggressive cancer that tends to metastasize to other parts of the body. The estimated numbers of new melanoma cases and deaths in Europe are 144.2 per 100,000 and 27.1 per 100,000, respectively [1]. Melanoma often arises from melanocytes in the skin that are frequently exposed to sunlight. Increased exposure to ultraviolet (UV) light is one of the causative risk factors for melanoma. UV radiation directly affects nucleotide base pairing in DNA, especially in pyrimidine bases which are especially vulnerable to chemical change by absorption of UV energy [2]. DNA damage induces cellular responses that result in several different outcomes including DNA repair, cell cycle arrest, modulation of DNA replication dynamics, altered gene expression, and cell death. When DNA repair processes fail, accumulated DNA damage in melanocytes induces melanoma.

For stage I and II melanoma, surgical resection is the standard treatment and improves long-term survival with a 5-year survival rate of over 90% [3]. On the other hand, stage III melanoma is highly recurrent even after locoregional resection and has a poor prognosis due to metastases. For metastatic melanoma, the standard treatment is pharmacological therapy. Currently, we have a wide range of treatment options such as chemotherapy with dacarbazine, BRAF inhibitors, MEK inhibitors, and immune checkpoint inhibitors. Dacarbazine was considered as a gold standard therapy for metastatic melanoma until a BRAF inhibitor and an anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) antibody were approved by the Food and Drug Administration (FDA) in 2011. The median survival time was 7.7 months for advanced metastatic melanoma patients treated with dacarbazine, which was approved by FDA in 1975 [4]. Dacarbazine is an alkylating chemotherapy drug which causes DNA modification. In order to achieve better responses with dacarbazine, combination treatment of dacarbazine

with other chemotherapies was evaluated. However these combination treatments induced high toxicity. In parallel to the work on dacarbazine improvement, genomic analysis of melanoma has been conducted.

Sequencing analysis focusing on the genes of the mitogen-activated protein kinase (MAPK) pathway revealed that BRAF somatic missense mutations are observed in 66% of malignant melanoma cases [5]. All mutations are located within the kinase domain and improve BRAF activity. Furthermore, mutated BRAF has oncogenic activity and can transform NIH3T3 cells. These findings led to the development of BRAF inhibitors as anti-melanoma drugs. A BRAF inhibitor, vemurafenib, is an orally available and selective inhibitor of the BRAF V600E mutant and was approved by the FDA for the treatment of patients with nonresectable or metastatic melanoma in 2011. A phase 3 study (BRIM-3) comparing vemurafenib to dacarbazine treatment in patients with previously untreated metastatic melanoma with BRAF V600E mutation showed that the overall survival rate after 6 months was 84% in the patients treated with vemurafenib and 64% in the patients treated with dacarbazine [6]. However, disease progression was observed in 50% of patients treated with BRAF inhibitors approximately 6 months after treatment initiation [6-8]. Resistance to MAPK pathway inhibitors is triggered by several mechanisms, such as alternative splicing of BRAF, BRAF amplification, RAS mutations, and activation of the phosphoinositide 3-kinase (PI3K)-AKT pathway [9-13]. One of the factors suggested as a key driver that makes melanomas resistant to MAPK pathway-targeted inhibitors is the upregulation of MITF [13-15].

Another genomic feature of malignant melanoma is a high tumor mutation burden (TMB). Mutation analysis of 7,042 primary cancers in 30 different tumor types revealed that malignant melanoma exhibited the highest mutation prevalence [16]. The high TMB in malignant melanoma triggers the increased neoantigen presentation on major histocompatibility complex

(MHC) molecules and induces immune response. Therefore, melanoma is thought to be sensitive to immune checkpoint inhibitor treatment. Indeed, immune checkpoint inhibitor treatment has shown clinical efficacy in patients with malignant melanoma. Ipilimumab binds CTLA-4, an immune checkpoint protein that downregulates pathways responsible for T cell activation and was approved by FDA in 2011. In a phase 3 study, ipilimumab improved the overall survival of patients previously treated metastatic melanoma [17]. Additionally, combination treatment of ipilimumab and dacarbazine improved the overall survival of patients with previously untreated metastatic melanoma [18]. The second approved immune checkpoint inhibitor in melanoma by FDA is pembrolizumab. Pembrolizumab is a humanized immunoglobulin G4 mAb targeting programmed cell death protein 1 (PD-1) molecule. In a phase 3 clinical study (Keynote-006 trial), pembrolizumab prolonged the progression-free survival and overall survival and had less high-grade toxicity when compared with ipilimumab in patients with advanced melanoma [19]. Furthermore, combination treatment of ipilimumab and nivolumab, an anti-PD-1 antibody, improved the median overall survival when compared with the treatment of ipilimumab alone [20]. Although combination treatment of anti-CTLA-4 antibody or anti-PD-1 antibody improved the efficacy compared with the monotherapies, disease progression was observed in 50% of patients 11.5 months after treatment initiation [20].

Treatment for metastatic melanoma has been improved by BRAF inhibitors and immune checkpoint inhibitors, but a subset of the patients is still insensitive to them. Therefore, novel drugs with other mechanisms of action are required. In order to explore novel anti-melanoma therapeutics, I screened a chemical library against a melanoma cell line panel and identified CH5552074, as described in Chapter I. Examination of the action mechanisms of the compound suggested that MITF suppression is the potential mechanism for melanoma cell growth inhibition.

MITF is a key transcription factor that is expressed in a melanocytic lineage and has a key role in melanocyte development, differentiation, and transformation [21, 22]. MITF activity is regulated by its upstream activators and suppressors and modulated at transcriptional, post-transcriptional, and post-translational levels [23]. In melanoma cells, genetic alterations in MITF are observed. Quantitative polymerase chain reaction (PCR) analysis revealed that MITF amplification was observed in 10% of primary cutaneous melanoma and 21% of metastatic melanoma cases [24]. Growth of melanoma cells with MITF copy gain was inhibited following MITF suppression with dominant-negative MITF introduction [24]. Genetic alterations in MITF also include single base mutation. A germline missense mutation in MITF (E318K) is observed at a significantly higher frequency in genetically enriched melanoma patients than in the controls [25, 26]. Because codon 318 in MITF is located in a small ubiquitin-like modifier (SUMO) consensus site, MITF E318K mutation severely impairs SUMOylation of MITF and enhances MITF function. Additionally, MITF activation is observed in melanoma cells resistant to BRAF inhibitors [13, 14]. This observation suggests that the combination of BRAF inhibition and MITF suppression exhibits enhanced growth inhibitory effects against melanoma cells. Therefore, I examined melanoma cell growth inhibition activity of the combination treatment of the BRAF inhibitor, PLX4720, and the MITF suppressor, CH6868398, as described in Chapter II.

Chapter I: MITF suppression by CH5552074 inhibits cell growth in melanoma cells

1. Introduction

Malignant melanoma is an aggressive type of cancer and has a poor prognosis for advanced disease. Selective molecular targeted therapy that blocks BRAF and MEK improves survival in melanoma patients with BRAF mutation remarkably [6, 27, 28], but the therapy rarely shows durable response due to various resistant mechanisms [13, 14]. In another therapeutic approach, antibodies that block CTLA-4 and PD-1 significantly improve overall survival in patients with melanoma [17, 29-31]. However, these immune checkpoint inhibitors are effective only in limited patients. Therefore, there is room to develop therapeutics with novel mechanisms of action on malignant melanomas.

A potential mechanism for melanoma therapy is to target MITF, which is expressed in melanomas and has been shown by various research studies to be a transcription factor with a critical role in melanocyte development and transformation [21, 22]. MITF activity induces cell proliferation and MITF expression levels are increased by mutant BRAF, which triggers constitutive hyperactivation of MEK/ERK signaling in melanoma cells [32]. Additionally, gene amplification of MITF is observed in up to 20% of melanomas [24], and MITF activation is observed in melanomas resistant to BRAF inhibitors [13, 14]. Finally, the finding that inhibition of MITF mRNA reduces cell growth in melanoma cells also supports the potential of MITF as a target for melanoma therapy [24].

In this study, I identified CH5552074 as an anti-melanoma agent in a skin cancer cell panel

and then used IPA to investigate the mode of action by which it induced inhibition of cell growth. Following the results of the IPA analyses, I also evaluated the MITF-dependent potential of this compound to induce cell growth inhibition in melanoma cells.

2. Materials and Methods

2.1. Reagents

CH5552074 was synthesized at Chugai Pharmaceutical Co., Ltd (Kanagawa, Japan). Dharmacon ON-TARGETplus siRNA was purchased from GE Healthcare (Buckinghamshire, UK).

2.2. Cell cultures

SK-MEL-5, SK-MEL-1, SK-MEL-28, A-431, SK-MEL-2, HMCB, and C32 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). A375 cells were purchased from DS Pharma Biomedical Co., Ltd (Osaka, Japan). A2058 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). SK-MEL-30 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All cell lines were cultured according to the supplier's instructions.

2.3. *In vitro* cell growth assay

The cells were seeded into 96-well plates containing various concentrations of the compound or siRNA with Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) and were incubated for 4 days at 37°C. The absorbance at 450 nm or the chemiluminescent signal for the cell viability was measured with a Microplate-Reader iMark (Bio-Rad Laboratories, Hercules, CA, USA) after addition of a Cell Counting Kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) or with an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA) after addition of a Cell Titer-Glo luminescence cell viability kit (Promega, Madison, WI, USA), respectively. Anti-proliferative activity was calculated using the formula

(1−T/C) × 100 (%), where T represents the measured values of reagent-treated cells and C represents that of untreated control cells.

For real-time analysis, IncuCyte Zoom System (Essen Biosciences, Ann Arbor, MI, USA) was utilized. The cells were seeded into 96-well plates and treated with various concentrations of CH5552074. The culture plate was incubated in an IncuCyte Zoom System in a cell culture incubator. Cell images were captured in 2 fields per well every 4 hours following treatment with CH5552074. The occupied area (% confluence) was determined using IncuCyte Zoom software (Essen Biosciences).

2.4. Microarray analysis

Total RNA was extracted from cells with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) and was reverse transcribed, labeled, and hybridized to Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. The gene expression level for each probe was calculated utilizing the guanine-cytosine robust multiarray analysis (GC-RMA) algorithm [33].

2.5. IPA analysis

IPA software [34] was used to identify upstream pathways that might affect CH5552074 sensitivity. Correlation factors of the probes were calculated from the natural logarithmic expression levels of each probe in a microarray analysis of 10 cell lines and natural logarithmic IC50 values calculated in an *in vitro* cell growth assay of the cell lines treated with CH5552074. To remove genes that were expressed in the 10 cell lines at almost the same level, probes with a natural logarithmic expression range of <2 across samples were filtered out. The gene set for IPA consisted of genes for which the correlation factors of the corresponding probes were >0.7

or <−0.7. The gene set was analyzed through IPA core analysis and an upstream analysis.

2.6. Western blot analysis

Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors and phosphatase inhibitors. Western blot analysis was performed as described previously [35]. Primary antibodies were used for MITF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, TX, USA). Signals were developed using Pierce Western Blotting Substrate Plus (Thermo Scientific, Rockford, IL) and detected with LAS-4000 (Fujifilm, Tokyo, Japan).

2.7. Gene expression analysis with Taqman probes

Total RNA was isolated from cells with an RNeasy Mini Kit (Qiagen). For real-time PCR (RT-PCR) analysis, the isolated RNA and TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA) were used. The following Taqman probes (Applied Biosystems) were used: MITF (Hs01117294_m1) and GAPDH (4325792). Reactions were run on a ViiA 7 Real-Time PCR System (Applied Biosystems). The gene expression levels of MITF were measured against the housekeeper gene GAPDH and determined utilizing ViiA 7 Software (Applied Biosystems).

3. Results

3.1. Identification of CH5552074 and its in vitro cell growth inhibition activity

To identify an anti-melanoma agent, I screened a chemical library against multiple melanoma cell lines and A-431 epidermal carcinoma in skin and found a lead compound, CH5552074, which had a novel chemical structure (Fig. 1-1a). In an *in vitro* cell growth inhibition assay, CH5552074 exhibited cell growth inhibition activity against the melanoma cell lines (Fig. 1-1b). Additionally, the *in vitro* cell growth inhibition activity of CH5552074 was examined to determine the sensitivity in melanoma cell lines with different BRAF mutation status: BRAF V600E mutation (SK-MEL-5, A375, SK-MEL-1, SK-MEL-28, A2058, and C32 cells) and wild-type BRAF (SK-MEL-30, A-431, SK-MEL-2, and HMCB cells). The IC50 values of CH5552074 were 0.24–1.2 μM in melanoma cell lines and were independent of BRAF mutation status (Table 1-1). These data suggested that CH5552074 had a different mode of action from BRAF inhibitors.

3.2. MITF modulation as a candidate for the mode of action of CH5552074

CH5552074 had cell growth inhibition activity against the melanoma and epidermal carcinoma cell lines, but the mode of action by which the compound showed this activity was unclear. In order to investigate the mode of action, I performed IPA of a gene set that correlated with CH5552074 sensitivity in each cell line. First, the mRNA expression profile in each cell line was examined by microarray analysis. I then calculated the correlation factors of each probe using its expression level and the IC50 values in each cell line and identified the 107 genes for which correlation factors of the corresponding probes were >0.7 or <−0.7 (Table 1-2). In order to determine an upstream regulator that is activated or inactivated in CH5552074-sensitive cell

lines, 107 genes were subjected to core and upstream analysis in IPA. These analyses suggested MITF, atrophin-1 (ATN1), forskolin, GSK0660, and tripartite motif-containing 24 (TRIM24) as candidates for upstream regulators of CH5552074 sensitivity in melanoma cells (Table 1-3). I focused on MITF as a regulator of CH5552074 sensitivity because it has an extremely low p-value within these candidates, which represents the highest probability. When mRNA expression levels of MITF and genes regulated by MITF (transient receptor potential cation channel, subfamily M, member 1 (TRPM1) [36] and solute carrier family 45 member 2 (SLC45A2) [37]) were assessed, they had a tendency to be higher in CH5552074-sensitive cell lines (Fig. 1-2). These observations suggested the possibility that CH5552074 modulated MITF function in melanoma cells.

3.3. MITF protein reduction induced by CH5552074

To investigate whether inhibiting MITF leads to cell growth inhibition in melanoma cells, the effect of MITF siRNAs on MITF inhibition was examined in a cell growth inhibition assay in melanoma cells. MITF siRNAs induced the degradation of MITF protein (Fig. 1-3a) and MITF mRNA (Fig. 1-3b) at 48 hours. In a further cell growth inhibition assay of SK-MEL-5 and SK-MEL-30 cells treated with 10 nM of siRNAs for 4 days, cell growth inhibition was observed (Fig. 1-3c), which suggests that the cell growth of SK-MEL-5 and SK-MEL-30 cells depends on MITF.

3.4. Cell growth inhibition induced by MITF knockdown in SK-MEL-5 cells

To evaluate the effect of the compound on MITF protein expression, I evaluated MITF protein levels in SK-MEL-5 and SK-MEL-30 cells that had been treated with CH5552074 for 24 hours at doses from 0.01 μM to 10 μM. The western blotting analysis showed that

CH5552074 treatment in melanoma cells suppressed MITF expression dose-dependently (Fig. 1-4a). When MITF expression levels from 6 hours to 48 hours in SK-MEL-5 cells were measured, expression levels of MITF increased in a time-dependent manner (Fig. 1-4b). MITF expression in SK-MEL-5 cells treated with 10 μM of the compound were suppressed from 24 hours. At 24 hours, the MITF expression level in the treated cells decreased to 36% that of the control cells. To examine whether MITF reduction leads to cell growth inhibition, I evaluated real-time cell growth inhibition in SK-MEL-5 cells treated with CH5552074 for 72 hours. Cell growth inhibition induced by the compound was observed from around 24 hours after the compound was added (Fig. 1-4c). These data support that cell growth inhibition in SK-MEL-5 cells treated with CH5552074 is correlated with MITF downregulation.

To support MITF specificity of CH5552074 effect, its activity in cell lines of other tumor types without MITF expression was evaluated. First, I determined MITF expression levels in the lung cancer cell line NCI-H345 and the colon cancer cell line SW1116. MITF expression was observed in melanoma cell lines, but not in NCI-H345 or SW1116 cells (Fig. 1-4d). Although CH5552074 showed cell growth inhibition activity in melanoma cells with MITF expression (Fig. 1-1b), cell growth inhibition was not observed in NCI-H345 and SW1116 cells when they were treated with CH5552074 (Fig. 1-4e), which suggested that CH5552074 exhibited cell growth inhibition activity in melanoma cells with MITF expression.

To ascertain whether the reduced MITF protein was induced by inhibited MITF mRNA expression, I then measured mRNA expression levels of MITF in cells treated with CH5552074 for 24 hours. Although MITF protein was reduced by the compound at 24 hours, no change in MITF mRNA expression was observed (Fig. 1-4f), which suggested that the reduction in MITF protein level induced by CH5552074 was regulated at the protein level, but not at the mRNA level.

4. Discussion

Although some potent therapeutics, such as trametinib, that were identified in a cell-based phenotypic screen have been approved and utilized in clinic [38, 39], the difficulty of identifying the target molecule of compounds obtained by this approach remains as a major hurdle in drug development. Although the chemical biology approach is commonly used, the success rate is not so high. The approach outlined in this paper successfully correlates the cellular sensitivity of a newly-identified agent with the mRNA expression profiles to identify the target molecule by pathway analysis and then confirms the activity in a western blotting analysis. In this study, I identified the mode of action of an anti-melanoma agent, CH5552074, which had been selected in a phenotypic screen, by correlating the cellular sensitivity to the compound with the mRNA expression profiles in IPA (Table 1-2 and 1-3). The suggestion that the MITF pathway may contribute to CH5552074 activity was then confirmed in a western blotting and an RT-PCR analysis that showed MITF protein degradation in melanoma cells treated with CH5552074 (Fig. 1-4).

MITF has a critical role in melanoma cell growth and seems to be an attractive molecular target for melanoma therapy. Overexpression of MITF protein and gene amplification of MITF are observed in melanoma patients [21, 24]. Moreover, when MITF-amplified melanoma cell lines are transduced with a dominant-negative MITF mutant, suppression of MITF inhibits melanoma cell growth [24]. MITF overexpression is observed in melanomas resistant to BRAF inhibitors and a knockdown of MITF restores the activity of a BRAF inhibitor in resistant melanoma cells [40]. However, the lack of a catalytic domain in MITF protein makes it difficult to obtain inhibitors that directly target MITF protein.

Because targeting MITF is an attractive strategy for melanoma therapy and overcoming

resistance to BRAF inhibitors but is difficult to achieve directly, current research is investigating the potential of inhibiting the MITF function indirectly. Chemical inhibition of AMP-activated kinase (AMPK) led to a decrease in MITF protein levels [41], and histone deacetylase (HDAC) inhibitors suppressed MITF mRNA expression in melanoma cells [42]. The human immunodeficiency virus type 1 (HIV1)-protease inhibitor nelfinavir suppressed MITF expression through paired box gene 3 (PAX3) inhibition and sensitized melanoma cells with BRAF mutation and NRAS mutation to MAPK pathway inhibitors [15]. This study contributes to this line of research by finding that CH5552074 reduced expression levels of MITF protein and induced cell growth inhibition in melanoma cells.

To fully understand the potential of the compound as a melanoma therapeutic, the mechanisms by which CH5552074 reduces MITF expression need to be investigated. Some mechanisms that modulate MITF expression levels have been reported. One example is the transcription factor PAX3, which was shown to regulate MITF transcription in melanoma cells [43]; however, CH5552074 suppressed MITF expression at the protein level but not at the transcription level (Fig. 1-4). In another example, expression levels of MITF were affected by mutant BRAF activation of the MAPK pathway and by inhibition of the pathway by BRAF inhibitors and MEK inhibitors [32, 44, 45]. To determine whether CH5552074 could affect the MAPK pathway, I evaluated its effect on modulating the phosphorylation levels of ERK, but no change was observed (data not shown). Further research will investigate the mechanisms by which CH5552074 reduces MITF expression to augment the current study.

5. Tables

Table 1-1 IC 50 values of CH5552074 and BRAF mutation status in skin cancer cell lines.

Cell lines	IC50 (μM)	BRAF
SK-MEL-5	0.24	V600E
SK-MEL-30	0.30	wt
A375	0.55	V600E
SK-MEL-1	0.58	V600E
SK-MEL-28	0.65	V600E
A-431	0.96	wt
SK-MEL-2	0.97	wt
A2058	1.1	V600E
HMCB	1.1	wt
C32	1.2	V600E

Melanoma cells and A-431 cells were incubated for 4 days at 37°C in the presence of serially diluted CH5552074. Cell viability was measured with Cell Counting Kit-8 solution.

Table 1-2 Gene set with correlation factors analyzed in IPA.

Gene name	R	Gene name	R	Gene name	R	Gene name	R	Gene name	R
NEFL	-0.90	GBAP1	-0.79	PLEKHH1	-0.75	ABCA5	-0.73	PVRL2	0.71
THEM4	-0.90	DMRT2	-0.79	WIPF3	-0.75	RAB38	-0.73	NMI	0.73
CCDC171	-0.89	TSPAN10	-0.79	ZC3HAV1L	-0.75	CHD1	-0.72	PODXL	0.73
MITF	-0.88	GALM	-0.78	STK4	-0.75	C1orf115	-0.72	NFE2L3	0.74
DACT1	-0.87	SPP1	-0.78	SLC16A7	-0.75	MET	-0.72	HEG1	0.74
NR4A3	-0.87	WDR17	-0.78	PRKCH	-0.75	LINC00883	-0.72	NUCKS1	0.75
TAF1C	-0.87	LPIN1	-0.78	GJA3	-0.75	SLCO5A1	-0.72	TM4SF1	0.75
OLFM1	-0.87	NADSYN1	-0.78	RGS20	-0.75	PDK4	-0.71	ENC1	0.75
MRPL21	-0.85	MRPL35	-0.77	SLC45A2	-0.74	PSPH	-0.71	CSRP1	0.76
ZFYVE26	-0.85	SLC5A4	-0.77	ABCC4	-0.74	TMEM251	-0.71	PLEC	0.76
CPVL	-0.84	IL12RB2	-0.77	SNX25	-0.74	HEY2	-0.71	HOXA3	0.78
RETSAT	-0.84	SLFN5	-0.77	FGD4	-0.74	CYSTM1	-0.71	CRIM1	0.79
SYT14	-0.84	SLC30A1	-0.77	NCS1	-0.74	R3HCC1L	-0.71	GBP1	0.79
SOCS2	-0.84	PRKD3	-0.76	SLC16A6	-0.74	C5orf22	-0.71	PTPRF	0.80
TEX41	-0.83	MCOLN1	-0.76	STARD10	-0.74	SEPSECS	-0.71	ATP1B1	0.81
DAB2	-0.83	CHMP1B	-0.76	C9orf91	-0.74	CHAC1	-0.71	AIM1	0.81
CTNS	-0.83	RNFT1	-0.76	ENTPD6	-0.73	LINC00473	-0.71	LOXL2	0.81
ITPKB	-0.82	SLC25A16	-0.76	TRIM63	-0.73	ATP13A2	-0.71	FLNB	0.87
USP36	-0.82	UGCG	-0.76	AMDHD2	-0.73	OCA2	-0.70		
IL6R	-0.82	SYTL5	-0.76	TMC6	-0.73	PLXNC1	-0.70		
MTURN	-0.81	HAGLR	-0.76	M6PR	-0.73				
TRPM1	-0.80	GPR161	-0.76	TRIB3	-0.73				
RASD1	-0.79	TRIO	-0.76	PNPLA3	-0.73				

R, correlation factor of natural logarithmic expression levels of each probe in microarray analysis and natural logarithmic IC50 values in cell lines.

Table 1-3 Upstream analysis in IPA

Upstream regulator	p-value of overlap	Target molecules in dataset
MITF	8.40E-09	AIM1, AMDHD2, FGD4, IL6R, ITPKB, MET, MITF, SLC45A2, TMC6, TMEM251, TRPM1
ATN1	2.93E-05	ATP1B1, NEFL, PTPRF, SOCS2, SPP1, TM4SF1
forskolin	3.42E-05	ATP1B1, DAB2, GBP1, MET, MITF, NR4A3, OCA2, PDK4, PTPRF, SLC45A2, SPP1, TM4SF1
GSK0660	5.79E-05	PDK4, TRIM63
TRIM24	7.15E-05	CSRP1, NMI, PLEC, SOCS2, SPP1

6. Figures

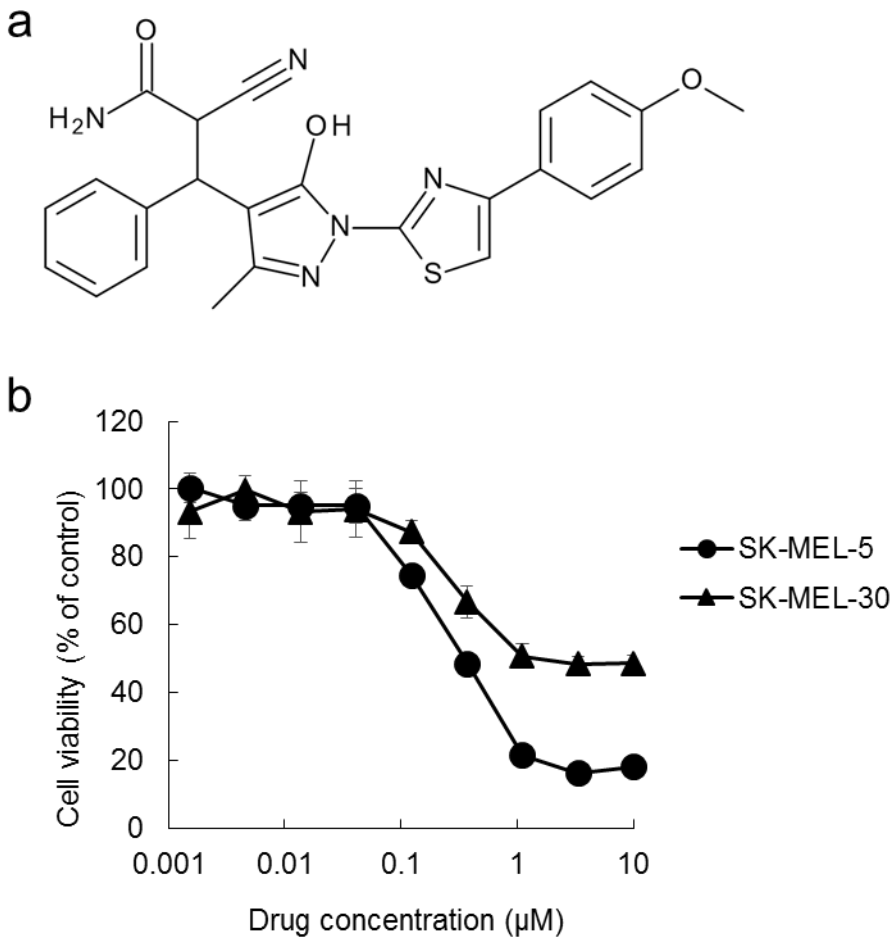


Fig. 1-1 Cell growth inhibition activity of CH5552074. (a) Chemical structure of CH5552074. (b) SK-MEL-5 and SK-MEL-30 cells were incubated for 4 days at 37°C in the presence of serially diluted CH5552074. Cell viability was analyzed with Cell Titer-Glo after 96-hour incubation. All points indicate the mean ± SD (n = 3).

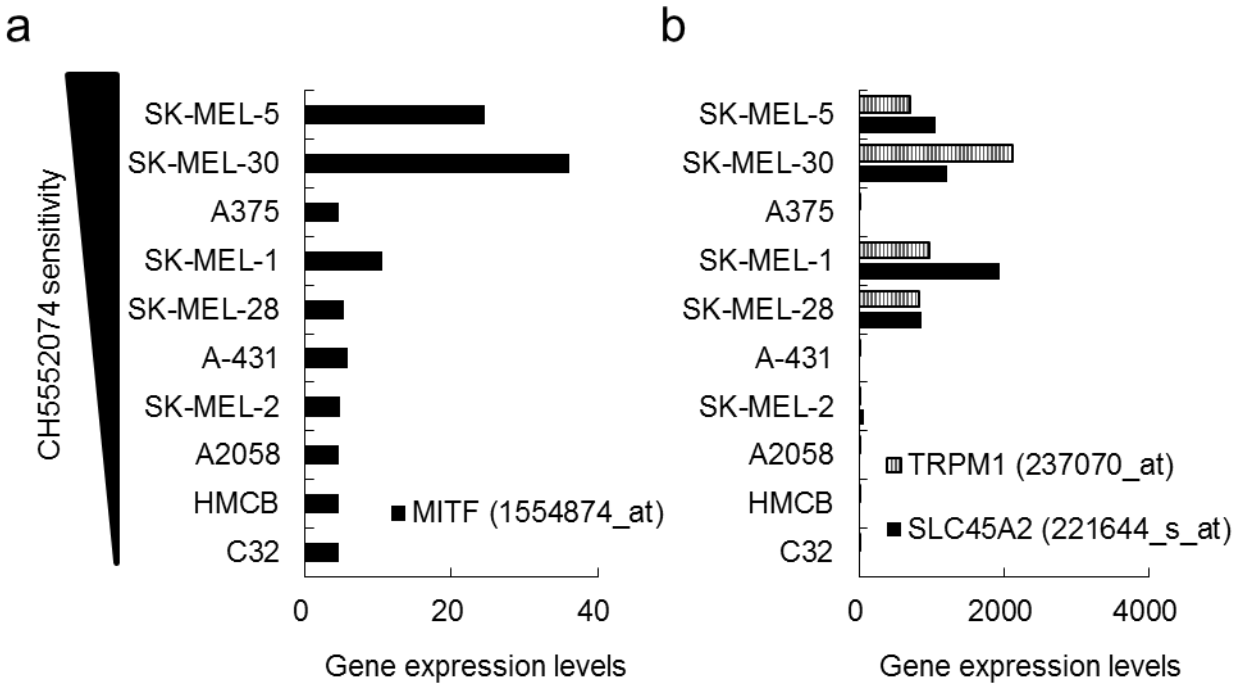


Fig. 1-2 Gene expression levels of MITF, TRPM1, and SLC45A2 in melanoma cell lines. Gene expression levels of (a) MITF and (b) TRPM1 and SLC45A2, which are regulated by MITF, were measured in a microarray analysis of RNA extracted from in vitro cultivated melanoma cell lines and A-431 cells.

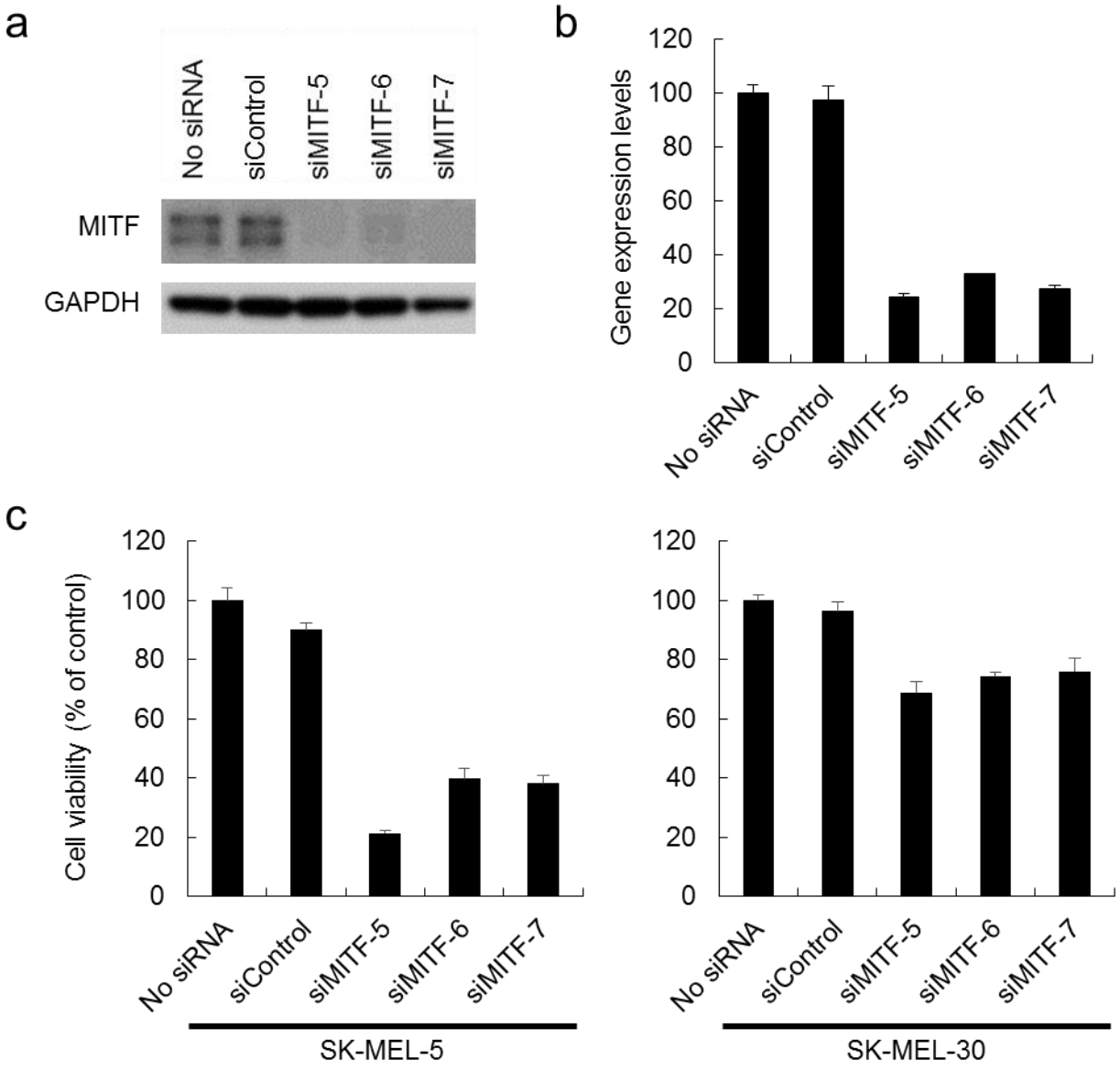


Fig. 1-3 Cell growth inhibition induced by MITF knockdown in melanoma cells. SK-MEL-5 cells were transfected with 10 nM of 3 clones of MITF siRNA or control siRNA and incubated for 48 hours before measuring (a) MITF protein levels by western blotting, and (b) MITF mRNA expression levels by RT-PCR. All points indicate the mean ± SD (n = 3). (c) Cell viability was analyzed with Cell Titer-Glo after 96-hour incubation. All points indicate the mean ± SD (n = 3).

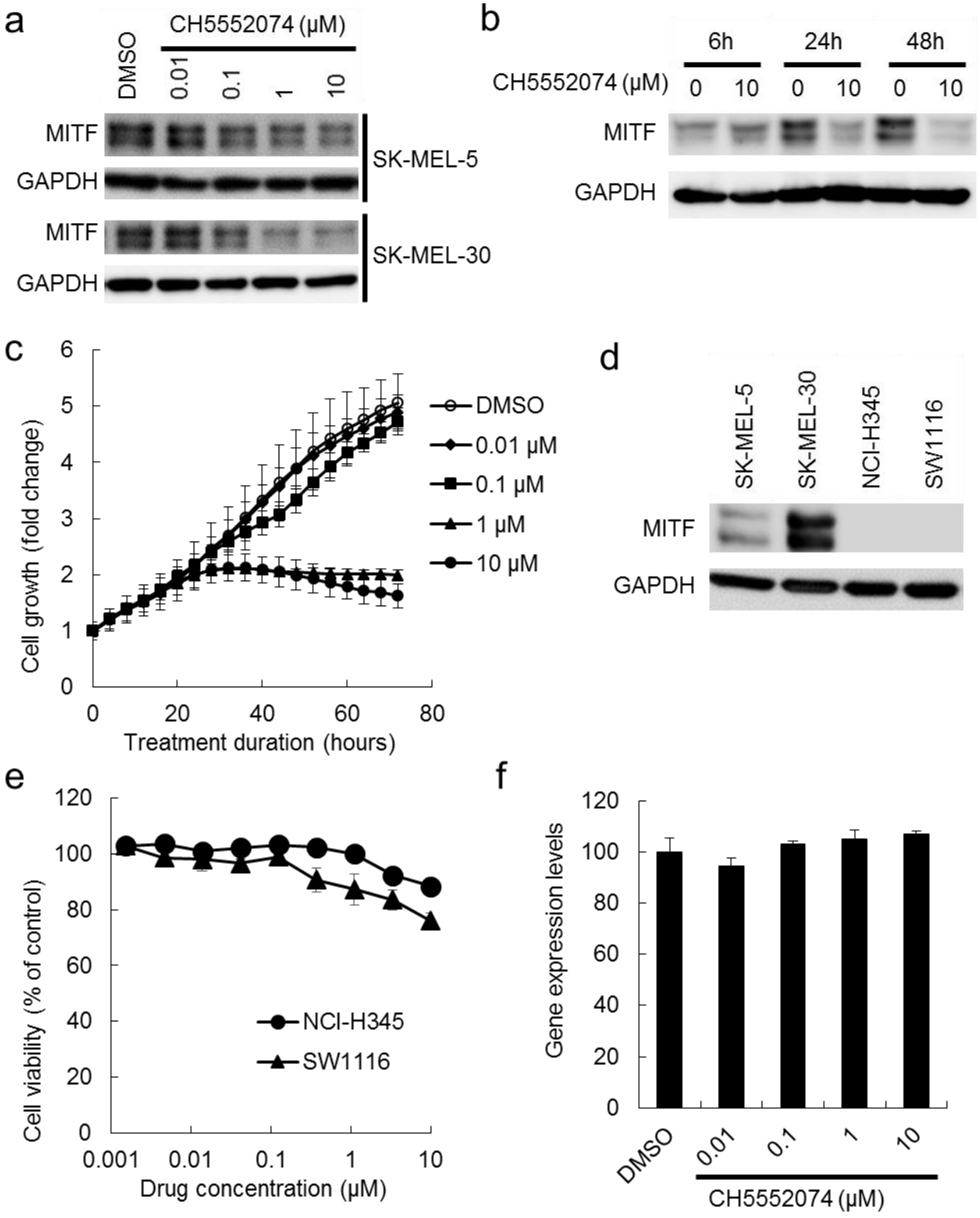

a
CH5552074 (μM)
DMSO
0.01
0.1
1
10
MITF
GAPDH
SK-MEL-5
MITF
GAPDH
SK-MEL-30
b
6h
24h
48h
CH5552074 (μM)
0
10
0
10
0
10
MITF
GAPDH
c
Cell growth (fold change)
Treatment duration (hours)
DMSO
0.01 μM
0.1 μM
1 μM
10 μM
d
SK-MEL-5
SK-MEL-30
NCI-H345
SW1116
MITF
GAPDH
e
Cell viability (% of control)
Drug concentration (μM)
NCI-H345
SW1116
f
Gene expression levels
DMSO
0.01
0.1
1
10
CH5552074 (μM)

Fig. 1-4 MITF protein reduction and cell growth inhibition induced by CH5552074 in melanoma cell lines. (a) Western blots show the protein expression levels of MITF in melanoma cells that had been treated with the indicated concentrations of CH5552074 for 24 hours. (b) SK-MEL-5 cells were cultivated for indicated hours with 10 μM of CH5552074. (c) In SK-MEL-5 cells incubated for 72 hours at 37°C in the presence of serially diluted CH5552074, real-time cell growth was monitored by cell images taken every 4 hours and assessed by IncuCyte Zoom System. All points indicate the mean ± SD (n = 3). (d) The expression levels of MITF protein in the indicated cell lines were evaluated by western blotting analysis. (e) NCI-H345 lung cancer cells and SW1116 colon cancer cells were incubated for 4 days at 37°C in the presence of serially diluted CH5552074. Cell viability was analyzed with Cell Titer-Glo after 96-hour incubation. All points indicate the mean ± SD (n = 3). (f) The expression levels of MITF mRNA in SK-MEL-5 cells incubated in the presence of serially diluted CH5552074 for 24 hours were analyzed by RT-PCR analysis. All points indicate the mean ± SD (n = 3).

Chapter II: MITF suppression improves the sensitivity of melanoma cells to a BRAF inhibitor

1. Introduction

Malignant melanoma is an aggressive type of cancer that is life-threatening in the advanced stage. Approximately 50% of cutaneous melanomas harbor BRAF mutations that cause constitutive activation of downstream signaling via the MAPK pathway [5, 46]. Although melanoma patients with the BRAF mutations are sensitive to selective BRAF inhibitors and combination therapy with MEK inhibitors [6, 27, 28], 50% of patients treated with BRAF inhibitors have disease progression approximately 6 months after treatment initiation [7, 8].

MITF is a melanocytic lineage-specific transcription factor that regulates the expression of various genes essential for melanin synthesis in melanocytes [47]. MITF is frequently expressed in melanoma and has a critical role in transformation and progression of melanoma [21, 22]. Moreover, genetic amplification of MITF is observed in melanomas, and MITF inhibition by dominant-negative MITF reduces cell growth in melanoma cells [13, 24, 48]. These findings suggest that MITF is a potential target for melanoma therapy.

Because targeting MITF directly with small molecules is significantly challenging, a potential strategy is pharmacologically inhibiting MITF indirectly. Support for this strategy is that HDAC inhibitors suppress MITF mRNA expression in melanoma cells by inhibiting SRY-related HMG-box 10 (SOX10) expression [42], and that the HIV1-protease inhibitor nelfinavir suppresses MITF expression by inhibiting PAX3 and also sensitizes melanoma cells with BRAF and NRAS mutations to MAPK pathway inhibitors [15]. Additionally, the MITF suppressor

CH5552074, which was previously identified by screening in a melanoma cell panel, was found to downregulate MITF protein and induce growth inhibition in melanoma cells [49].

In this study, I describe CH6868398, an orally available MITF suppressor derivatized from the CH5552074 compound. I evaluated the activity of CH6868398 *in vitro* and *in vivo* and, moreover, examined the effect of combination treatment with a selective BRAF inhibitor, PLX4720.

2. Materials and Methods

2.1. Reagents

CH6868398, CH6813089, and CH6987687 were synthesized at Chugai Pharmaceutical Co., Ltd (Kanagawa, Japan). PLX4720 was purchased from Abcam (Cambridge, UK). Dharmacon ON-TARGETplus siRNA was purchased from GE Healthcare (Buckinghamshire, UK). siRNA transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA).

2.2. Cell cultures

SK-MEL-5, SK-MEL-28, and A101D cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MEL-HO, IPC-298, and SK-MEL-30 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). SK-MEL-5 and SK-MEL-28 cells were maintained in EMEM supplemented with 10% FBS and 1 mM sodium pyruvate. MEL-HO, IPC-298, and SK-MEL-30 cells were maintained in RPMI-1640 supplemented with 10% FBS. A101D cells were maintained in DMEM supplemented with 10% FBS and 1 mM sodium pyruvate.

2.3. *In vitro* cell growth assay

The cells were seeded onto 96-well plates at 3000 cells/well containing various concentrations of the compound and were incubated for 4 days at 37°C. The chemiluminescent signal for cell viability was measured with an EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA) after addition of a Cell Titer-Glo luminescence cell viability kit (Promega, Madison, WI, USA). Anti-proliferative activity was calculated using the formula (1−T/C) ×

100 (%), where T represents the measured values of reagent-treated cells and C represents that of untreated control cells.

2.4. Western blot analysis

Cells were seeded in a 6-well plate at 2 x 10^5 cells/well for single compound treatment or 3 x 10^5 cells/well for combination treatment, and were treated with inhibitors or a solvent control (0.1% dimethyl sulfoxide (DMSO)) for 2, 6, 24, or 48 hours. For siRNA studies, cells were seeded in a 6-well plate at 2 x 10^5 cells/well and were treated with siRNAs. After siRNA treatment for 24 hours, cells were treated with inhibitors or a solvent control (0.1% DMSO) for 24 hours. Cells were lysed in Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) containing protease inhibitors and phosphatase inhibitors. Cell lysates were denatured with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific, Rockford, IL, USA) with 2-mercaptoethanol. At least 10 μg of cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed as described previously [35]. Primary antibodies were used for MITF (Santa Cruz Biotechnology, Dallas, TX, USA, #sc-56725), GAPDH (Abcam, #ab201822), phospho-ERK1/2 (Cell Signaling Technology, #9101), ERK1/2 (Cell Signaling Technology, #4696 and #9102), cleaved PARP (Cell Signaling Technology, #5625), cleaved Caspase 3 (Cell Signaling Technology, #9664), and Bim (abcam, #ab32158). Signals were developed using Pierce Western Blotting Substrate Plus (Thermo Fischer Scientific) and detected with LAS-4000 (Fujifilm, Tokyo, Japan). The bands were quantified by densitometry using ImageQuant TL 1D software (GE Healthcare, Little Chalfont, UK) and normalized using the expression levels of GAPDH protein.

2.5. Mouse xenograft studies

All animal experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd (approval number: 12-292). All animals were housed in a specific pathogen-free environment under controlled conditions. Chlorinated water and irradiated food were provided ad libitum. The health of the mice was monitored daily. A total of 5 x 10^6 SK-MEL-30 cells/200 μL per mouse were subcutaneously injected into the right flank of female BALB/c nu/nu mice (Charles River Laboratories, Kanagawa, Japan). Tumor size was measured using a gauge twice a week, and tumor volume (TV) was calculated using the following formula: TV = $ab^2/2$, where a is the length of the tumor and b is the width. Once the tumors reached a volume of approximately 200 mm^3, mice were randomized into groups (n = 4 in each group), and treatment was initiated. Vehicle (10% DMSO, 10% Cremophor EL, 15% polyethylene glycol, and 15% 2-hydroxypropyl-β-cyclodextrin (HPCD) in distilled water) or CH6868398 was orally administered by gavage once daily for 10 days. Tumor growth inhibition (TGI) was calculated using the following formula: TGI = $(1 - [T_t - T_0]/[C_t - C_0]) \times 100$, where T (TV of the treated group, T_0 on the first day of treatment or T_t on Day t) and C (TV of the control group, C_0 on the first day of treatment or C_t on Day t) represent mean tumor volume. The maximum tolerated dose (MTD) was defined as the dose that resulted in neither lethality nor more than 20% bodyweight loss.

2.6. Gene expression analysis with TaqMan probes

Cells were seeded in a 6-well plate at 2 x 10^5 cells/well and were treated with inhibitors or a solvent control (0.1% DMSO) for 48 hours. Total RNA was isolated from cells with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA). For RT-PCR analysis, 200-1,000 ng of the isolated RNA and TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City,

CA, USA) were used. The following TaqMan probes (Applied Biosystems) were used: SLC45A2 (Hs01125486_m1), TRPM1 (Hs00931865_m1), and GAPDH (4325792). Reactions were run on a ViiA 7 Real-Time PCR System (Applied Biosystems) with 40 amplification cycles. The gene expression levels were measured against the housekeeper gene GAPDH and determined utilizing ViiA 7 software (Applied Biosystems).

2.7. Statistical analysis

Statistical analyses were performed using JMP version 11.2.1 software (SAS Institute, Cary, NC, USA). Statistical significance was determined by Dunnett's test, Student's t-test, or Tukey's test and is shown in the figure legends. $P < 0.05$ was considered to indicate a statistically significant result. Significant p values are labeled with asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. Identification of CH6868398 and its activity on cell growth inhibition

To obtain an orally available anti-melanoma drug, I derivatized MITF suppressor CH5552074, which had been identified in a screening for compounds in a melanoma cell panel [49], and identified CH6813089 (Fig. 2-1a). Because CH6813089 is a racemic mixture of CH6868398 and CH6987687, I evaluated the activity of each of the three compounds in SK-MEL-5 cells, which are sensitive to siRNAs against MITF (Fig. 2-2). In the cell growth inhibition assay, CH6813089 and CH6868398 were more potent than CH6987687 (Fig. 2-1b), which suggested that CH6868398 was the active compound included in CH6813089. When MITF suppression activity of CH6868398 and CH6987687 in SK-MEL-5 cells was examined, MITF expression levels significantly decreased in the cells treated with CH6868398 (Fig. 2-1c).

3.2. CH6868398 activity on cell growth inhibition and MITF suppression in melanomas

To further evaluate cell growth inhibition by CH6868398 in melanoma cells, I utilized 6 melanoma cell lines with different BRAF mutation status: BRAF V600E mutation (MEL-HO, SK-MEL-5, SK-MEL-28, and A101D cells) and wild-type BRAF (IPC-298 and SK-MEL-30 cells). First, I examined MITF expression and whether cell growth depended on MITF expression in the melanoma cell lines. MITF expression was observed in MEL-HO, SK-MEL-5, SK-MEL-28, IPC-298, and SK-MEL-30 cells, but not in A101D cells (Fig. 2-3a). When I treated the melanoma cells with 3 individual siRNAs against MITF, cell proliferation of the melanoma cells with MITF expression was inhibited by 3 siRNAs against MITF (Fig. 2-2). Then I assessed the cell growth inhibition activity of CH6868398 in melanoma cells with and

without MITF expression. CH6868398 showed significant cell growth inhibition activity against MEL-HO, SK-MEL-5, SK-MEL-28, IPC-298, and SK-MEL-30 cells which had MITF expression and growth dependence on MITF, but not against A101D cells which had no MITF expression (Fig. 2-3b). This cell growth inhibition activity was not related to BRAF mutation status and did not correlate to MITF protein expression levels, but was related to sensitivity to MITF siRNAs (Fig. 2). To confirm the anti-tumor activity of CH6868398 against melanomas, I evaluated *in vivo* efficacy of the compound in a SK-MEL-30 xenograft mouse model. Daily oral administration of CH6868398 at MTD, 50 mg/kg, resulted in significant anti-tumor activity in the SK-MEL-30 model with a maximum TGI of 78% at Day 32 (Fig. 2-3c). In the *in vivo* efficacy study, the doses of CH6868398 tested were well tolerated without body weight loss, change in food intake, and death.

Next, to confirm whether CH6868398 suppressed MITF in CH6868398-sensitive melanoma cells, I treated melanoma cells with CH6868398 for 24 hours at doses from 0.01 μM to 10 μM, and found that MITF expression was suppressed by CH6868398 dose-dependently and was significantly downregulated with 10 μM of CH6868398 (Fig. 2-4a). Although mutated BRAF plays a critical role in regulating MITF expression [32], CH6868398 suppressed MITF expression not only in melanoma cells with BRAF mutation but also in those without BRAF mutation. When I measured MITF expression levels from 6 to 48 hours in melanoma cells treated with 10 μM of CH6868398, expression levels of MITF were significantly suppressed from 6 hours in SK-MEL-5 cells and from 24 hours in SK-MEL-30 cells (Fig. 2-4b). To address inhibition of the MITF function, I assessed the mRNA expression levels of SLC45A2 and TRPM1, which are regulated by MITF [36, 37], in melanoma cells treated with CH686838, and observed that CH6868398 significantly decreased these expression levels (Fig. 2-4c). These observations suggested that CH6868398 suppressed the MITF function and inhibited the cell

growth in melanoma cells.

3.3. Enhancement of cell growth inhibition activity of a BRAF inhibitor in combination with CH6868398 in melanoma cells with BRAF mutation

Because treatment with a selective BRAF inhibitor is standard therapy for melanoma patients with the BRAF mutations, I assessed the possible contribution of CH6868398 to selective BRAF inhibitor treatment in melanoma cells. First, I examined melanoma cell sensitivity to the BRAF inhibitor PLX4720, and found that PLX4720 dose-dependently inhibited cell growth in MEL-HO, SK-MEL-5, SK-MEL-28, and A101D cells (Fig. 2-5a). When I evaluated the expression levels of phospho-ERK in melanoma cells treated with PLX4720, PLX4720 decreased phospho-ERK levels (Fig. 2-5b). Then I analyzed MITF expression levels in melanoma cells treated with PLX4720, and found that the expression level of MITF protein was significantly decreased by PLX4720 treatment in SK-MEL-28 and MEL-HO cells, but not inhibited in SK-MEL-5 cells (Fig. 2-5b).

Because PLX4720 treatment did not suppress MITF expression levels in SK-MEL-5 cells, I confirmed whether MITF inhibition by CH6868398 enhances the cell growth inhibition activity of a BRAF inhibitor. To do this, I compared cell growth inhibition in melanoma cell lines by CH6868398 and/or PLX4720 treatment. In single agent treatment, PLX4720 showed more potent cell growth inhibition activity than CH6868398 in MEL-HO and SK-MEL-28 cells, but not in SK-MEL-5 cells (Fig. 2-6a). Compared with PLX4720 treatment, combination treatment of CH6868398 and PLX4720 showed more potent cell growth inhibition activity and significantly suppressed MITF expression levels in MEL-HO and SK-MEL-5 cells (Fig. 2-6a-b). This result indicated that MITF inhibition by CH6868398 enhanced the cell growth inhibition activity of PLX4720. To understand the mechanism of the CH6868398 and PLX4720

combination, I evaluated apoptosis induction after combination treatment. Combination treatment of CH6868398 and PLX4720 resulted in significantly enhanced levels of cleaved PARP and cleaved Caspase 3 in melanoma cells (Fig. 2-6b). Phosphorylation levels of ERK were not changed by combination treatment compared with PLX4720 treatment. When I evaluated the expression levels of Bim in melanoma cells treated with CH6868398 and PLX4720, combination treatment for 24 hours significantly induced Bim expression (Fig. 2-6c). These results suggest that CH6868398 is a potential combination partner for BRAF inhibitors in melanomas with BRAF mutations. Finally, to confirm that the combination effect of CH6868398 and PLX4720 was achieved through MITF suppression by CH6868398, the combination effect of PLX4720 and siRNAs against MITF was evaluated. As expected, MITF suppression by siRNAs significantly enhanced the levels of cleaved PARP and cleaved Caspase 3 induced by PLX4720 in SK-MEL-5 cells (Fig. 2-6d), which suggested that MITF suppression is a potential option for combination treatment with BRAF inhibitors in melanomas.

4. Discussion

MITF is an attractive target for melanoma treatment due to its oncogenic activity in melanomas. Gene amplification of MITF was observed in up to 20% of melanomas, and MITF inhibition by dominant-negative MITF resulted in cell growth inhibition in MITF-amplified melanoma cells [24, 50, 51]. Melanoma cell lines had elevated levels of MITF and were dependent on MITF activity for cell proliferation (Fig. 2-2). Moreover, indirect pharmacologic inhibition of MITF led to cell growth inhibition in melanoma cells [15, 42]. I also identified the MITF suppressor CH5552074 in a melanoma cell panel screen of a chemical library [49], and then generated an orally available MITF suppressor, CH6868398. The compound showed not only *in vitro* cell growth inhibition activity, but also *in vivo* efficacy in a SK-MEL-30 melanoma xenograft model without significant body weight loss (Fig. 2-3).

MITF expression is upregulated by MAPK pathway activation and can be modulated in melanomas by treatment with a MAPK pathway inhibitor. In melanoma cells, mutated BRAF upregulated MITF transcription by activating the MAPK pathway [32]. However, the relationship of MAPK pathway activation and MITF expression level is complex because activation of MAPK pathway also induces MITF degradation [52]. In melanoma cell lines with BRAF mutation, PLX4720 treatment significantly suppressed MITF expression in MEL-HO and SK-MEL-28 cells, but not in SK-MEL-5 cells (Fig. 2-5b). Although MITF expression levels after PLX4720 treatment are inconsistent among these melanoma cell lines, their cell growth activity is dependent on MITF (Fig. 2-2). Moreover, overexpression of MITF was observed in the early phase of resistance to BRAF inhibitors, and its suppression by siRNA inhibited cell proliferation in melanoma cells [15]. In clinic, overexpression of MITF through gene amplification was observed in melanomas that relapsed after BRAF inhibitor treatment

[13]. These observations suggested the idea that MITF inhibition by CH6868398 would enhance the cell growth inhibition activity of MAPK pathway inhibitors.

BRAF inhibitors are standard treatment for melanoma patients with BRAF mutations. Although the BRAF inhibitor PLX4720 exhibited cell growth inhibition activity to melanoma cell lines with BRAF mutation, MITF expression was still observed in the melanoma cells after PLX4720 treatment (Fig. 2-5), which suggests that MITF suppression by CH6868398 would lead to additional cell proliferation inhibition. CH6868398 treatment significantly suppressed MITF expression levels and cell growth in MEL-HO and SK-MEL-5 cells and induced apoptosis in the melanoma cells when the treatment was combined with PLX4720 treatment (Fig. 2-6a-c). Additional evaluation such as *in vivo* efficacy studies are required to confirm the potential of this compound as a therapeutic against melanomas with BRAF mutations.

PLX4720 is an analogue of vemurafenib which is widely used in clinic as a BRAF inhibitor for melanomas with BRAF mutation. The IC_{50}s of PLX4720 and vemurafenib on enzyme activity of BRAF V600E are 13 nM and 30 nM, respectively [53, 54]. The pharmacokinetics of vemurafenib used in clinic show that after continuous twice-daily dosing of 960 mg for 15 days mean plasma concentration remained stable and was about 100 μM [55, 56]. From this I can assume that the PLX4720 concentration (10 μM) seen in these combination studies with CH6868398 is reasonable.

CH6868398 treatment suppressed MITF protein in melanoma cell lines, but the mechanism of MITF protein suppression is still unclear. MITF expression is regulated by MAPK pathway activation, such as mutant BRAF activation, and is inhibited by BRAF inhibitors in some melanoma cell lines that have the BRAF mutation. A BRAF inhibitor inhibited phosphorylation of ERK in BRAF-mutant melanoma cells, but CH6868398 did not (Fig. 2-6b). In another example that may illustrate the cause of MITF suppression, the HIV-protease inhibitor

nelfinavir suppresses MITF expression by inhibiting PAX3, which is a transcription factor regulating MITF expression [15]. Nelfinavir treatment inhibits MITF mRNA expression, but CH6868398 did not suppress MITF mRNA in melanoma cells (Fig. 2-7). Determining the mechanisms of different effects on MITF suppression by CH6868398 is an important future task and will reveal the potential of the compound in melanoma therapy.

5. Figures

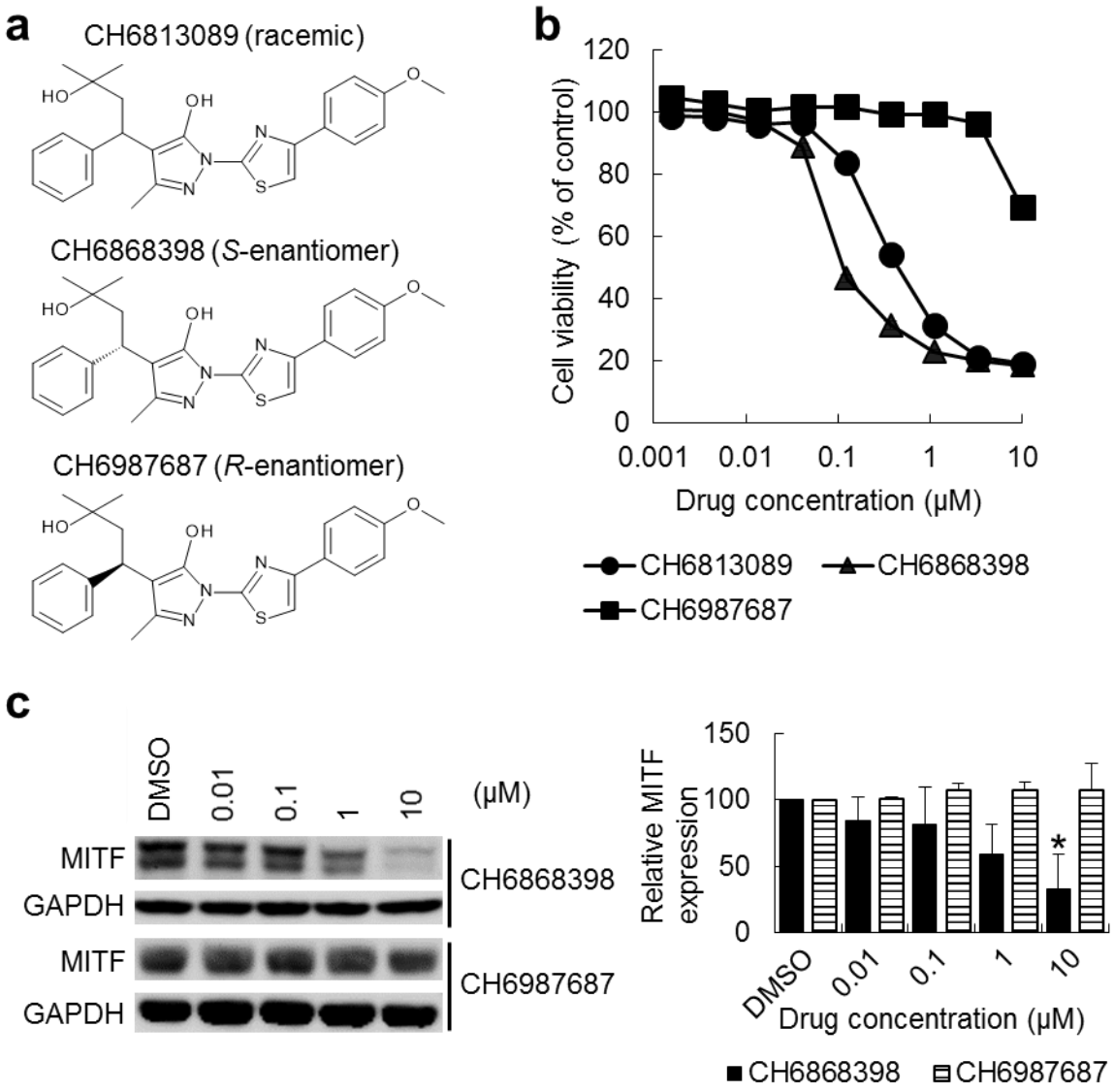

a
CH6813089 (racemic)
CH6868398 (S-enantiomer)
CH6987687 (R-enantiomer)
b
Cell viability (% of control)
Drug concentration (μM)
CH6813089
CH6868398
CH6987687
c
DMSO
0.01
0.1
1
10
(μM)
MITF
GAPDH
CH6868398
CH6987687
Relative MITF expression
Drug concentration (μM)

Fig. 2-1 CH6868398 activity on cell growth inhibition and MITF suppression. (a) Chemical structures of CH6813089, CH6868398, and CH6987687. (b) Cell growth inhibition activity of CH6813089, CH6868398, and CH6987687 in SK-MEL-5 cells. SK-MEL-5 cells were incubated for 4 days at 37°C in the presence of serially diluted compounds. Cell viability was analyzed with Cell Titer-Glo. All points indicate the mean ± SD (n = 3). (c) CH6868398 suppression of MITF in SK-MEL-5 cells. Melanoma cells were treated with the indicated concentrations of CH6868398 or CH6987687 for 24 hours. MITF protein levels were determined by western blotting (left) and densitometry analysis of the blots (right). The results shown are representative of the three independent experiments. Dunnett's test: compared with DMSO group.

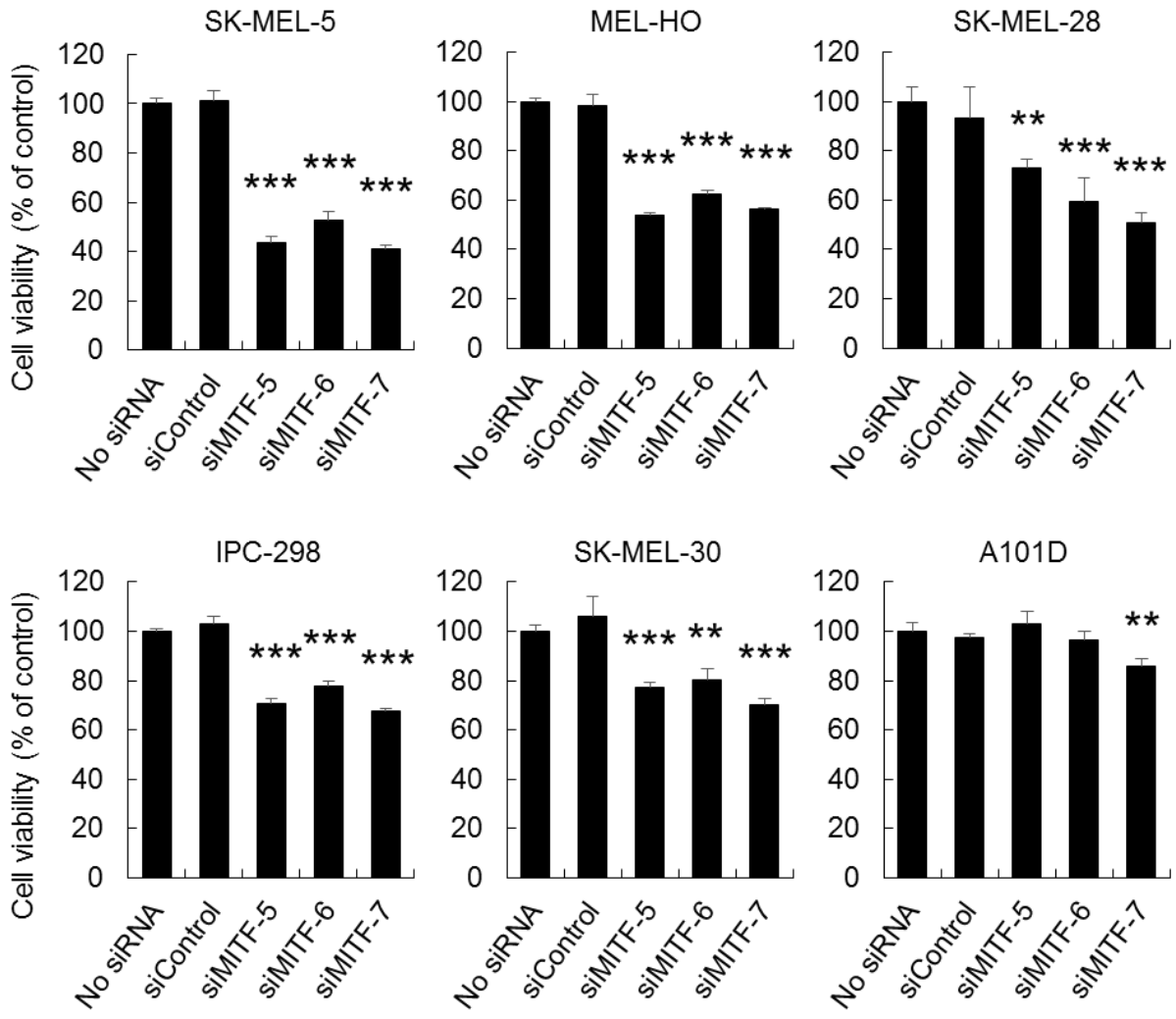


Fig. 2-2 MITF dependency of melanoma cell lines. Melanoma cells were transfected with 10 nM of 3 individual siRNAs against MITF or control siRNA and incubated for 4 days. Cell viability was analyzed with Cell Titer-Glo. All points indicate the mean ± SD (n = 3). Statistical significance compared with No siRNA group was examined using Dunnett's test.

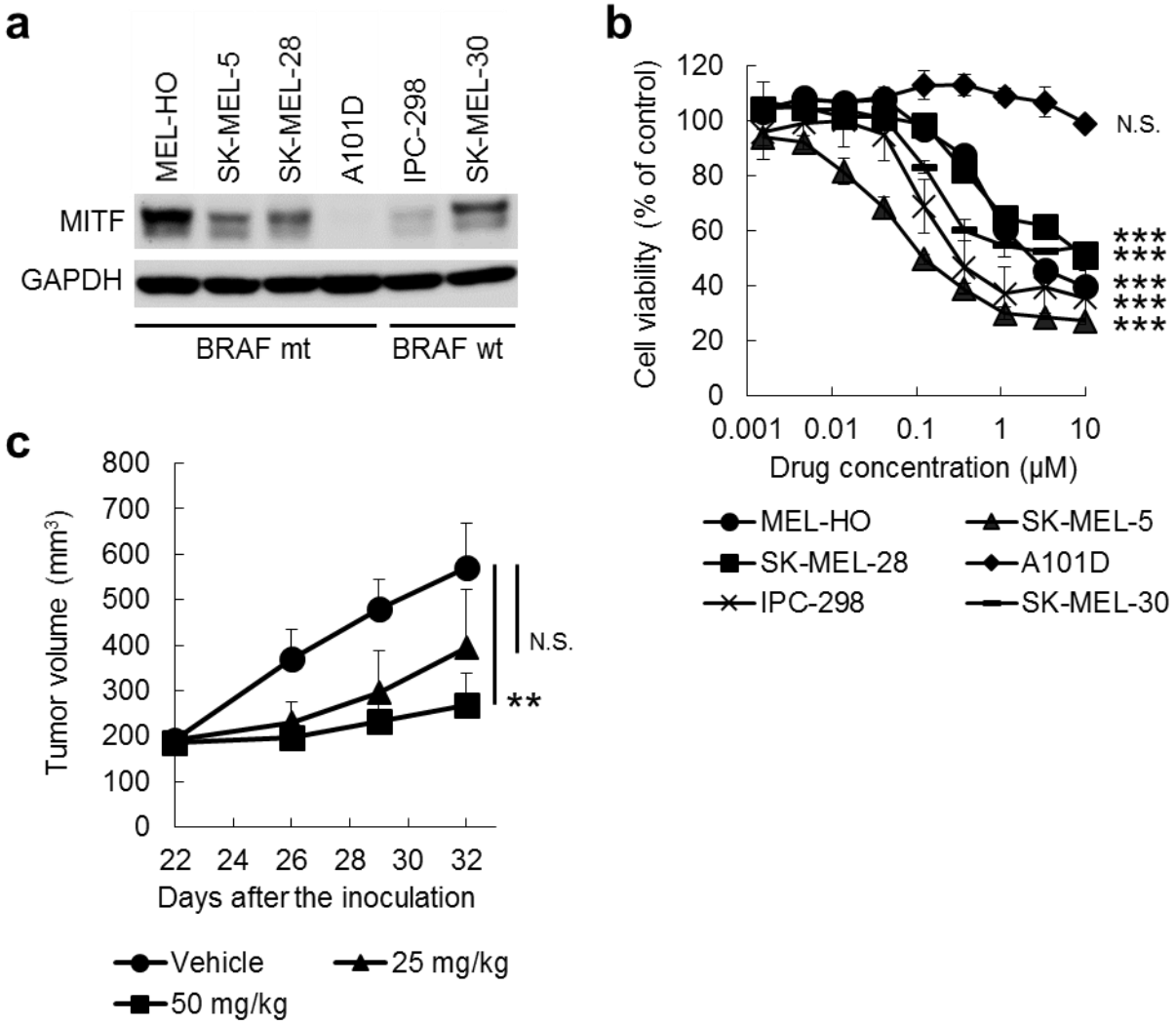
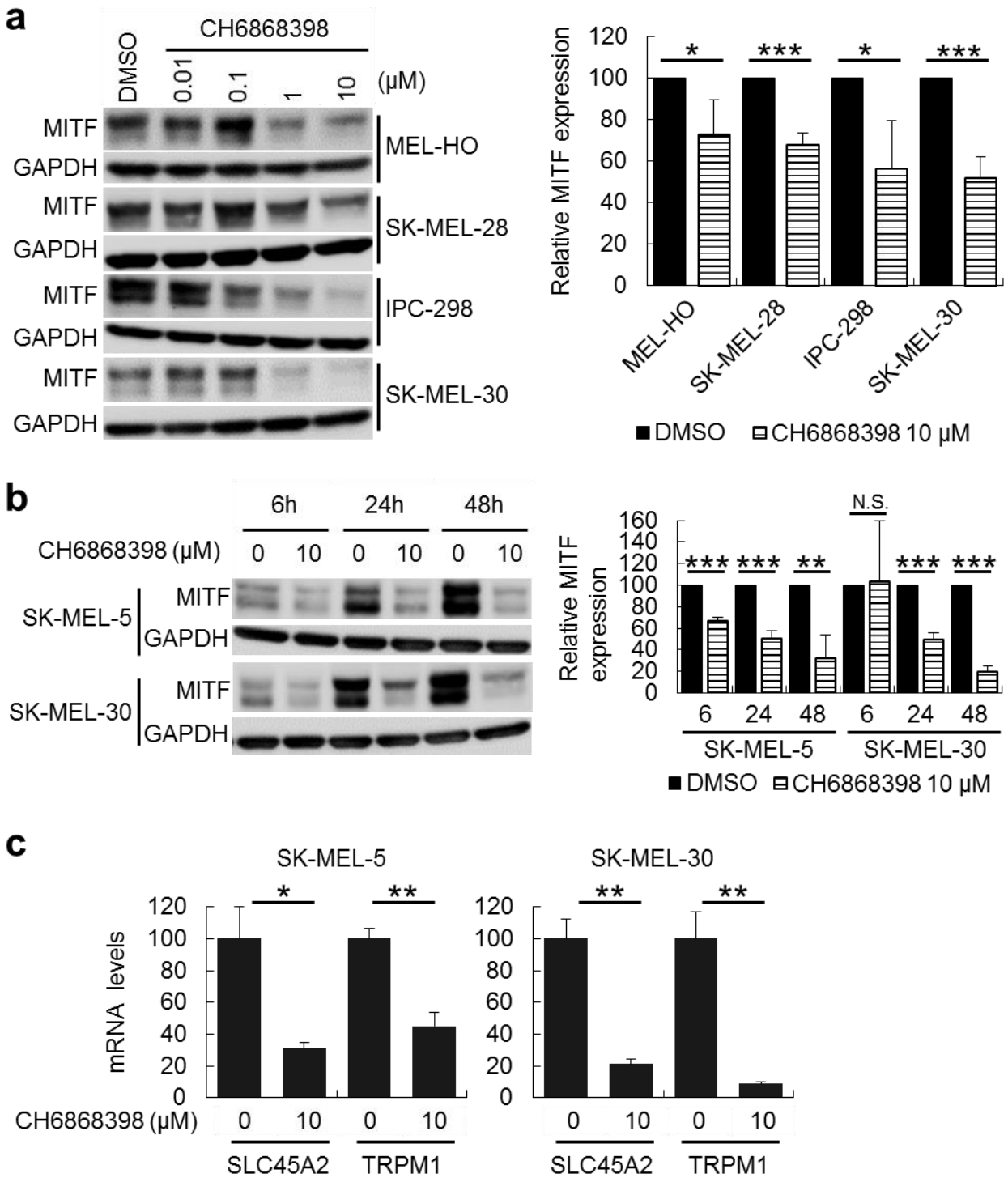


Fig. 2-3 Anti-tumor activity of CH6868398 in melanoma cells. (a) MITF protein expression levels in the melanoma cells, examined by western blotting. (b) Cell growth inhibition activity of CH6868398 in melanoma cell lines. The melanoma cell lines were incubated for 4 days at 37°C in the presence of serially diluted compounds. Cell viability was analyzed with Cell Titer-Glo. All points indicate the mean ± SD (n = 3). Student's t-test: compared with DMSO control group. N.S., not significant. (c) Efficacy of CH6868398 in a SK-MEL-30 melanoma xenograft model (n = 4). Mice inoculated with SK-MEL-30 cells were treated with CH6868398 orally once daily for 10 days at the indicated doses. Tumor volume in each group was measured. Data were shown as mean + SD. Dunnett's test: compared with vehicle treatment group at the final day.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DMSO
CH6868398
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0.1
1
10
(μM)
MITF
GAPDH
MEL-HO
SK-MEL-28
IPC-298
SK-MEL-30
Relative MITF expression
120
100
80
60
40
20
0
*
***
*
***
MEL-HO
SK-MEL-28
IPC-298
SK-MEL-30
DMSO
CH6868398 10 μM
b
6h
24h
48h
CH6868398 (μM)
0
10
0
10
0
10
SK-MEL-5
SK-MEL-30
MITF
GAPDH
Relative MITF expression
160
140
120
100
80
60
40
20
0
N.S.
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6
24
48
6
24
48
SK-MEL-5
SK-MEL-30
DMSO
CH6868398 10 μM
c
SK-MEL-5
SK-MEL-30
mRNA levels
120
100
80
60
40
20
0
*
**
**
**
CH6868398 (μM)
0
10
0
10
SLC45A2
TRPM1

Fig. 2-4 MITF suppression activity of CH6868398 in melanoma cells. (a) MITF suppression activity of CH6868398 in melanoma cells. Melanoma cells were treated with the indicated concentrations of CH6868398 for 24 hours. MITF protein levels were determined by western blotting (left) and densitometry analysis of the blots (right). The blot data shown are representative of the three independent experiments. Student's t-test: compared with DMSO treatment group. (b) Time course effects of CH6868398 on MITF suppression. The melanoma cell lines were cultivated for indicated hours with 10 μM of CH6868398 (left). The relative band intensities of MITF were quantified by densitometry (right). The blot data shown are representative of the three independent experiments. Student's t-test: compared with DMSO treatment group. (c) mRNA expression levels in the melanoma cells treated with 10 μM of CH6868398. mRNA expression levels were determined by RT-PCR analysis of the melanoma cells treated with the compound for 48 hours. All points indicate the mean ± SD (n = 3). Student's t-test: compared with DMSO treatment group.

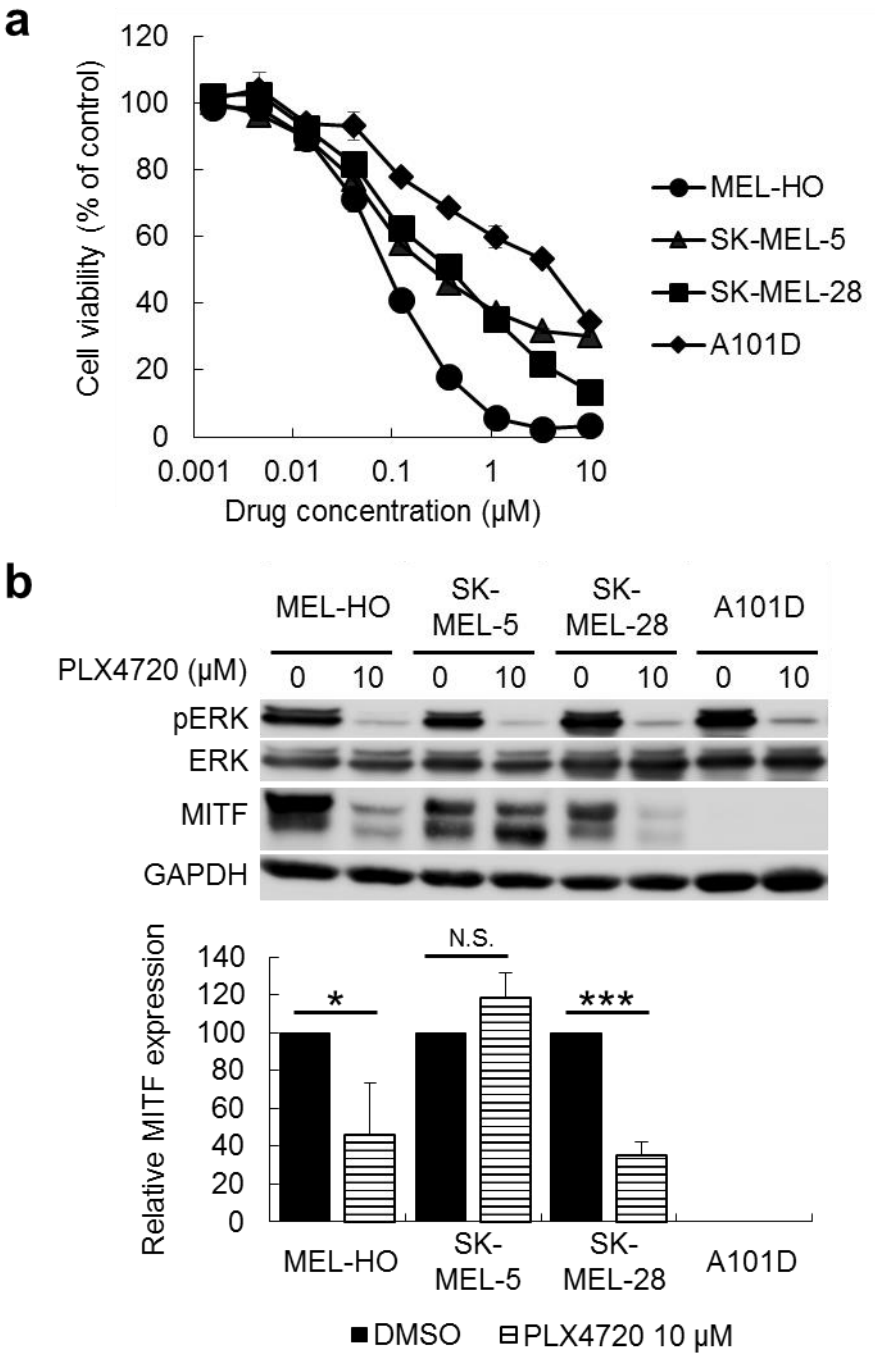
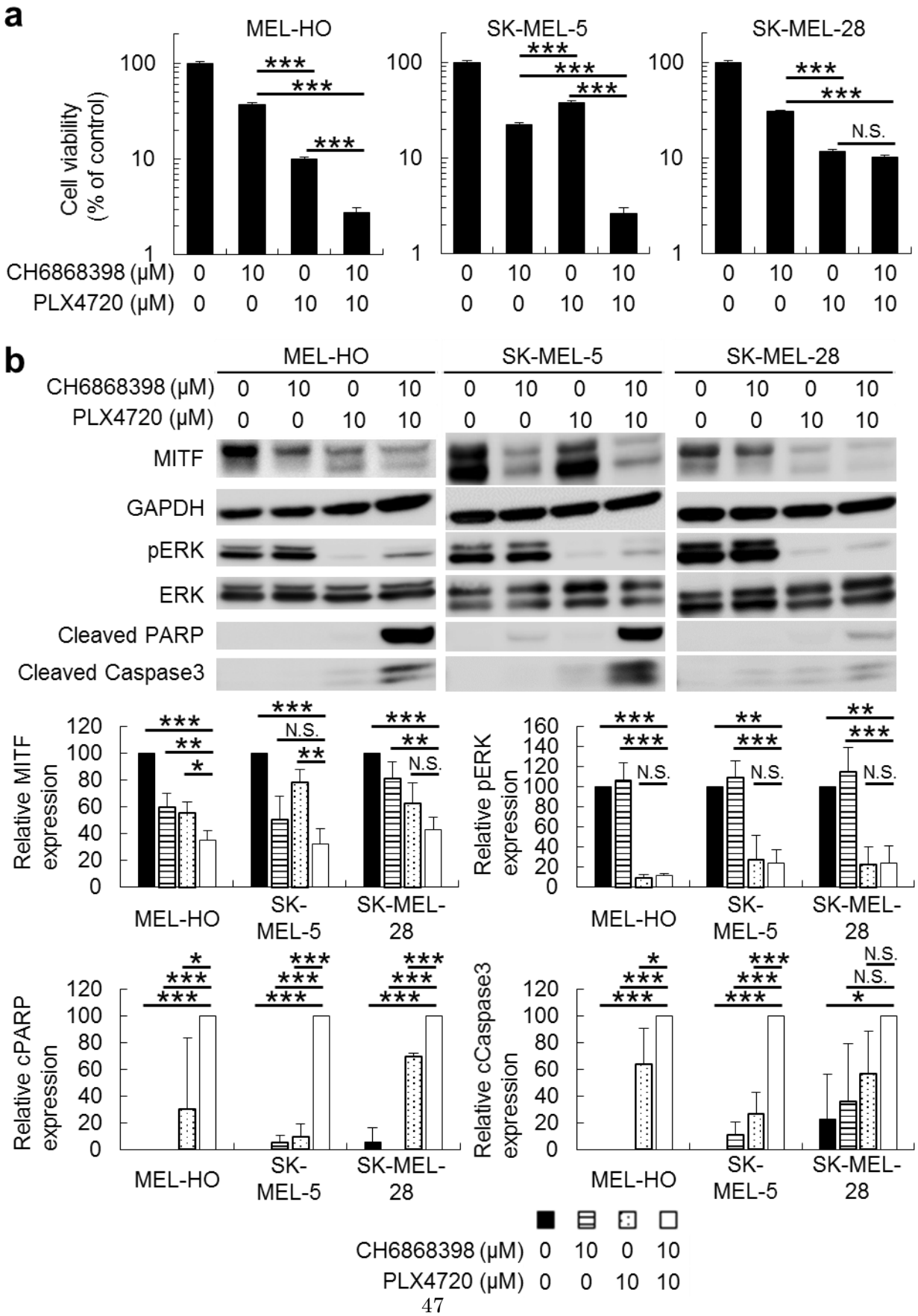

a
120
100
80
60
40
20
0
Cell viability (% of control)
0.001
0.01
0.1
1
10
Drug concentration (μM)
MEL-HO
SK-MEL-5
SK-MEL-28
A101D
b
MEL-HO
SK-MEL-5
SK-MEL-28
A101D
PLX4720 (μM)
0
10
0
10
0
10
0
10
pERK
ERK
MITF
GAPDH
Relative MITF expression
140
120
100
80
60
40
20
0
*
N.S.
***
MEL-HO
SK-MEL-5
SK-MEL-28
A101D
DMSO
PLX4720 10 μM

Fig. 2-5 Effect of PLX4720 on MITF expression and cell growth inhibition in melanoma cells. (a) Cell growth inhibition activity of PLX4720. The melanoma cell lines were incubated for 4 days at 37°C in the presence of serially diluted compounds. Cell viability was determined with Cell Titer-Glo. All points indicate the mean ± SD (n = 3). (b) Expression levels of phospho-ERK and MITF in melanoma cells treated with PLX4720. The melanoma cell lines with BRAF mutation were cultivated for 24 hours with 10 μM of PLX4720. The protein expression levels were examined by western blotting (top) and by densitometry (bottom). The blot data shown are representative of the three independent experiments. Student's t-test: compared with DMSO treatment group.


a
MEL-HO
SK-MEL-5
SK-MEL-28
Cell viability (% of control)
100
10
1
***
N.S.
CH6868398 (μM) 0 10 0 10
PLX4720 (μM) 0 0 10 10
b
MITF
GAPDH
pERK
ERK
Cleaved PARP
Cleaved Caspase3
Relative MITF expression
Relative pERK expression
Relative cPARP expression
Relative cCaspase3 expression
MEL-HO
SK-MEL-5
SK-MEL-28

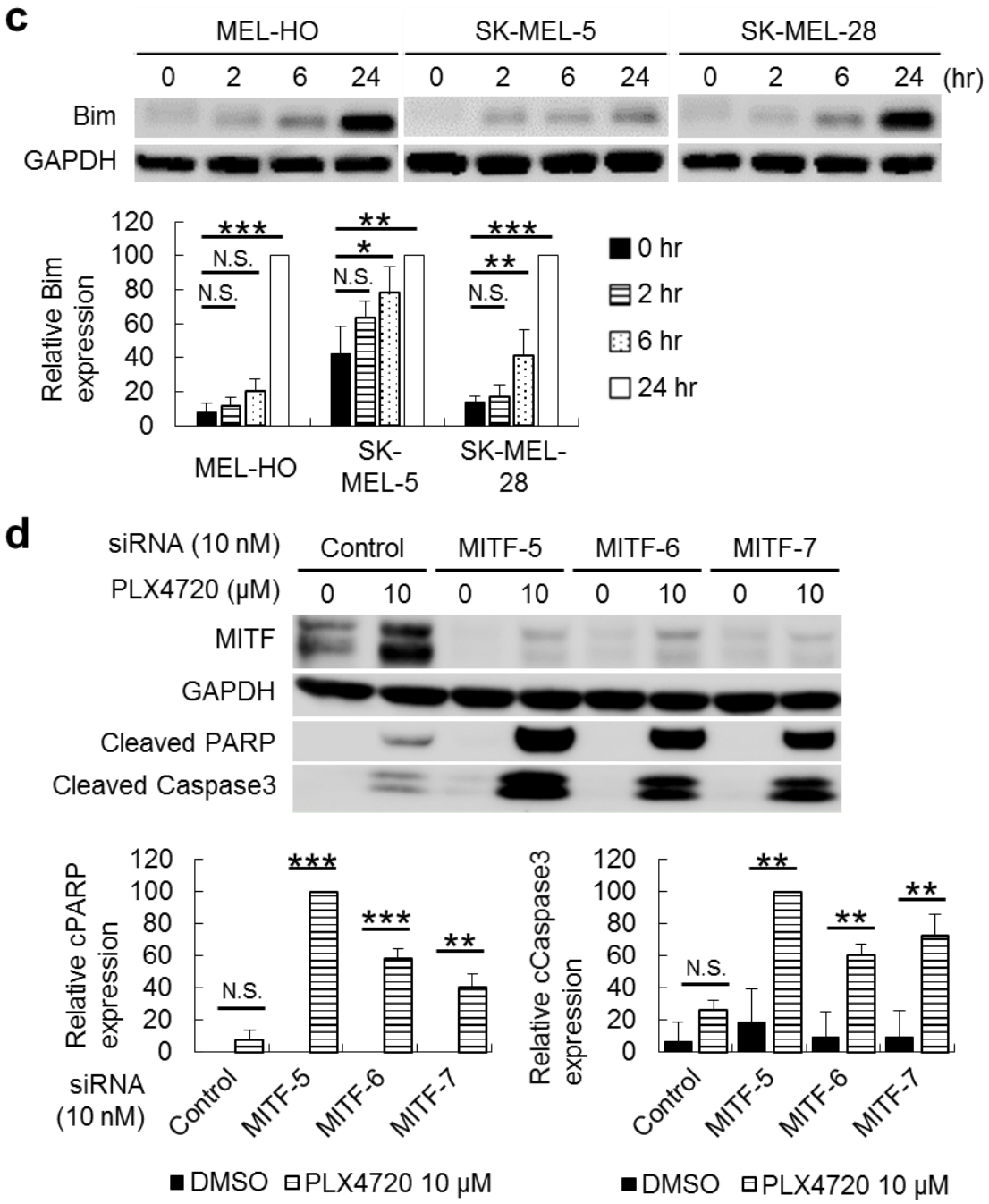

c
MEL-HO
SK-MEL-5
SK-MEL-28
0 2 6 24 0 2 6 24 0 2 6 24 (hr)
Bim
GAPDH
Relative Bim expression
120
100
80
60
40
20
0
***
N.S.
N.S.
**
*
N.S.
***
**
N.S.
0 hr
2 hr
6 hr
24 hr
MEL-HO
SK-MEL-5
SK-MEL-28
d
siRNA (10 nM)
Control
MITF-5
MITF-6
MITF-7
PLX4720 (μM)
0 10 0 10 0 10 0 10
MITF
GAPDH
Cleaved PARP
Cleaved Caspase3
Relative cPARP expression
120
100
80
60
40
20
0
N.S.
***
***
**
siRNA (10 nM)
Control
MITF-5
MITF-6
MITF-7
DMSO
PLX4720 10 μM
Relative cCaspase3 expression
120
100
80
60
40
20
0
N.S.
**
**
**
Control
MITF-5
MITF-6
MITF-7
DMSO
PLX4720 10 μM

Fig. 2-6 Combination effect of CH6868398 and PLX4720. (a) Cell growth inhibition activity of CH6868398 in combination with PLX4720. The melanoma cell lines were treated with 10 μM of CH6868398 and/or 10 μM of PLX4720 for 4 days. Cell viability was detected with Cell Titer-Glo. All points indicate the mean ± SD (n = 3). Statistical significance was determined using Tukey's test. (b) MITF suppression and apoptosis induction activity of CH6868398 in combination with PLX4720. The melanoma cell lines were cultivated for 24 hours with 10 μM of CH6868398 and/or 10 μM of PLX4720. The protein expression levels were detected using western blotting (top) and quantified by densitometry (bottom). The blot data shown are representative of the three independent experiments. Dunnett's test: compared with combination treatment group. (c) Bim induction activity of CH6868398 in combination with PLX4720. The melanoma cell lines were cultivated for indicated hours with 10 μM of CH6868398 and PLX4720 (top). The relative band intensities of Bim were determined by densitometry (bottom). The blot data shown are representative of the three independent experiments. Dunnett's test: compared with 0 hr group. (d) Apoptosis induction by BRAF inhibition and MITF suppression. SK-MEL-5 cells were cultivated for 24 hours with 10 μM of PLX4720 24 hours after treatment with 3 individual siRNAs against MITF (top). The relative band intensities were quantified by densitometry (bottom). The blot data shown are representative of the three independent experiments. Student's t-test: compared with DMSO treatment group.

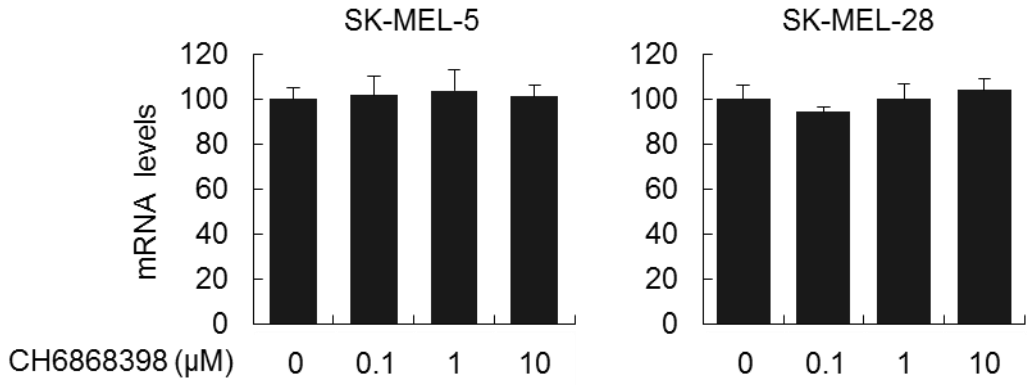


Fig. 2-7 MITF mRNA levels in cells treated with CH6868398. SK-MEL-5 and SK-MEL-28 cells were treated with the indicated concentrations of CH6868398 for 24 hours. MITF mRNA levels were determined by RT-PCR analysis. TaqMan probes for MITF and GAPDH were used. All points indicate the mean ± SD (n = 3). All groups compared with control group were not significant in Dunnett's test.

General Discussion

Melanoma treatment with BRAF inhibitors and immune checkpoint inhibitors significantly improves response rate and overall survival, but a subset of melanoma patients is still insensitive to these therapeutics. For such patients, a novel anti-melanoma agent with other action mechanisms is required.

Chapter I describes how I initially screened a small molecule compound library to identify compounds that inhibited the growth of melanoma cells using a melanoma cell panel [49]. One of the screening methods in drug discovery is target-based screening. When 15 first-in-class oncologic drugs approved between 1999 and 2013 were examined, it was found that 11 (73%) originated from target-based screens [38]. Target-based screens have been actively conducted in our laboratory. Specific inhibitors, such as an FGFR inhibitor and an ALK inhibitor, have been identified [57, 58]. One of the advantages of a target-based screening system is that millions of drug-like molecules in a chemical library are screened in a few months. Therefore, target-based screens are powerful screening systems for identification of hit compounds when critical target molecules are reported or identified. However, no promising target molecules which inhibition leads to tumor growth arrest have not been identified for malignant melanoma, except for mutated BRAF. Therefore, I selected a cell-based phenotypic screen for the identification of anti-melanoma agents.

One of the major hurdles in cell-based phenotypic screens in drug development is the identification of action mechanisms of hit compounds. For the identification of binding molecules to the hit compounds, a chemical biology approach is one of the effective strategies. One successful example for the identification of a binding molecule is the MEK inhibitor, JTP-70902. A target molecule of JTP-70902, which was identified as a $p15^{INK4b}$ mRNA inducer in

a cell-based phenotypic screen, was successfully identified as MEK1/2 by compound-immobilized affinity chromatography [39]. A key factor for success in the identification of the target molecule using a chemical biology approach is the potent activity of JTP-70902. Treatment with 10 nM of JTP-70902 induces $p15^{INK4b}$ expression, cell cycle arrest, and phosphorylation inhibition of ERK1/2 [39]. In contrast, IC_{50} values for cell growth inhibition of CH5552074 are greater than 100 nM (Table 1-1), and so, the chemical biology approach is not suitable for the identification of target molecules of CH5552074. Accordingly, IPA analysis is conducted for action mechanism examination.

IPA upstream analysis can suggest upstream factors that can affect the expression profile of dataset genes. In order to utilize IPA upstream analysis, I initially selected dataset genes, which were differentially expressed in CH5552074-sensitive skin cancer cell lines and CH5552074-insensitive cell lines (Table 1-2), and then the dataset genes were analyzed in IPA upstream analysis. The analysis suggested that MITF might regulate the expression levels of dataset genes (Table 1-3). To confirm the suggestion, western blotting analysis of MITF in the melanoma cells treated with CH5552074 was conducted. This assay showed that CH5552074 suppressed MITF expression (Fig. 1-4). A key success factor of this analysis is using the dataset genes generated from the gene expression data and the CH5552074 sensitivity data only of skin cancer cell lines because MITF is specifically expressed in melanoma. Actually, although I conducted the same analysis with dataset genes for cell lines in other tumor types, MITF was not suggested. This analysis may be a potential option for exploring the mechanism of action of a compound obtained in cell-based phenotypic screens.

Chapter II describes the effect of the combination of a BRAF inhibitor and an MITF suppressor, CH6868398. BRAF inhibitor monotherapy is effective against metastatic melanoma with BRAF mutation, but the progression-free survival was approximately 6 months

after treatment initiation [7, 8]. In order to improve the efficacy of BRAF inhibitors in patients with BRAF-mutated melanoma, combination treatment with MEK inhibitors has been examined in the clinic. As a result, progression-free survival was improved to 11.0 months in the COMBI-d study [59] and 11.4 months in the COMBI-v study in patients treated with a combination of dabrafenib, a BRAF inhibitor, and trametinib, a MEK inhibitor [60]; it was extended to 9.9 months in patients receiving combined treatment of vemurafenib, a BRAF inhibitor, and cobimetinib, a MEK inhibitor [28]. For further improvement of the efficacy of combination treatment with BRAF inhibitors, combination treatment of BRAF inhibitors with immune checkpoint inhibitors has been evaluated in early phase trials, which suggest that potential combination partners of BRAF inhibitors are still required.

In this study, it was found that melanoma cells still expressed MITF after BRAF inhibitor treatment (Fig. 2-5b). In such melanoma cases, additional MITF suppression by CH6868398 treatment would enhance cell growth inhibition effect. To confirm this possibility, MEL-HO and SK-MEL-5 cells were treated with PLX4720 and CH6868398, and as expected, additional MITF suppression and cell growth inhibition were observed (Fig. 2-6a-b). Moreover, combination treatment of PLX4720 and CH6868398 induced apoptosis in MEL-HO and SK-MEL cells (Fig. 2-6b-c). These results suggest that CH6868398 for MITF suppression is a potential combination partner with BRAF inhibitors in melanoma.

Focal genomic amplification of MITF is observed in melanoma cells resistant to BRAF inhibitors [13]. MITF overexpression is also found in BRAF inhibitor-resistant cell line models [15, 61] . In these models, MITF suppression leads to cell growth inhibition or delays drug resistance. Drug resistance to BRAF inhibitor is one of the critical issues in melanoma treatment. Therefore, further studies of CH6868398 with BRAF inhibitor-resistant models could address the issue of BRAF inhibitor resistance.

Finally, CH6868398 suppresses MITF activity and inhibits growth inhibition of melanoma cells (Fig. 2-3-4). Moreover, it showed anti-tumor activity in an *in vivo* melanoma xenograft model (Fig. 2-3c). Although MITF is a critical factor for proliferation of melanoma cells (Fig. 2-2), there are only a few reports on indirect MITF inhibitors [15, 41, 42]. Additional examination of action mechanisms of MITF suppression by CH6868398 is required, but the compound is a potential anti-melanoma therapeutic.

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